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PRINCIPAL INVESTIGATOR: Jae J. Song, Ph.D.

CONTRACTING ORGANIZATION: University of Pittsburgh
Pittsburgh, Pennsylvania 15260

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| 13. Abstract (<i>Maximum 200 Words</i>) (<i>abstract should contain no proprietary or confidential information</i>) We employed a glucose oxidase (GOD) to deplete glucose in the tumor. TRAIL cytotoxicity is potentiated in the presence of GOD. <i>In vitro</i> and <i>in vivo</i> studies have demonstrated that GOD has potent tumoricidal activity. Hydrogen peroxide produced by GOD is effective in preventing tumor growth, and its effect can be enhanced by hydrogen peroxide decomposition inhibitors such as 3-aminotriazole. Moreover, by comparison to normal cells, tumor cells are more susceptible to GOD. The combination of TRAIL and GOD effectively increased cell death compared to cells treated with TRAIL alone or GOD alone. We further examined the effect of GOD on TRAIL-induced caspase activation. TRAIL-induced activation of caspase was promoted by treatment with GOD. Glucose oxidase enhances TRAIL-induced cytotoxicity. We are currently building on this observation by developing a novel TRAIL-based gene therapy in conjunction with GOD. | | | | |
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Introduction :

Prostate cancer has become the most frequently diagnosed cancer among men in the United States. Early diagnosis and new surgical, hormonal, chemical and radiotherapy regimens have contributed to improved survival and quality of life for prostate cancer patients over the past ten years (Hanks et al., 1997; Keyser et al., 1997; Kupelian et al., 1997).

Approximately 25% to 60% of patients develop elevated prostate-specific antigen levels within 5 years following treatment (Vincini et al., 1997). When prostate cancers progress to an advanced stage, they are difficult to cure. Because tumor response and treatment morbidity depend upon tumor stage, there is a need to improve treatment effectiveness for these advanced tumors while controlling morbidity. Although conventional therapies have and will continue to play major roles in the treatment of prostate cancer, greater intervention will be required to significantly enhance primary local control of prostate cancer. One such approach is through the use of gene therapy techniques to correct errant characteristics of cancerous cells, or to specifically eliminate the cells through toxic gene expression (Roth et al., 1997).

Cancer gene therapy using cytotoxic genes has attracted great attention as one of the strategies treating cancers. For successful administration of gene therapy in cancer, the therapeutic gene should be delivered specifically to tumor cells and produced gene products that act only toxic to tumor cells without killing normal cells. On the basis of this premise, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a good candidate as a therapeutic gene due to its toxicity to tumors. TRAIL is an apoptosis-inducing member of the tumor necrosis factor (TNF) gene family (Wiley et al., 1995; Pitti et al., 1997). Recently, it has been shown that TRAIL is nontoxic systemically and that could slow the growth and in some cases, induce regression of tumor cell xenografts (Walczak et al., 1999). Preclinical studies in mice and primates have shown that administration of TRAIL can induce apoptosis in human tumors, but that no cytotoxicity to normal

organ or tissue is found (Walczak et al., 1999). Obviously, differential sensitivity between normal and tumor cells to TRAIL and the mechanism of TRAIL-induced apoptosis needs to be further studied (Gura, 1997; Ashkenazi et al., 1999; Keane et al., 1999).

Body :

We had tried several times for making the replication-incompetent adenoviral vector containing the CMV promoter-driven TRAIL gene as a first step for preparation of a replication-competent adenoviral vector containing TRAIL and Herpes simplex virus 1 thymidine kinase (HSV-TK) gene. However, even though successful construction of the adenoviral shuttle vector containing TRAIL gene, this type of vector was proven to be not replicating in host cell because of cytotoxic TRAIL expression which makes host cell death prior to the production of progeny virus. As a cytotoxic gene instead of TK gene, we adopted glucose oxidase gene (GOD). The reason for selecting GOD as a cytotoxic gene is as follows.

It is well known that abnormalities of the tumor vasculature result in an insufficient blood supply and development of a characteristic tumor microenvironment (hypoxia, low extracellular pH, high lactate concentration, and low glucose concentration). These characteristic features can affect the therapeutic response. Recently we reported that low glucose promotes TRAIL-induced apoptotic death (Nam et al., 2002). Similar results were obtained from experiments shown in Figures 1 and 2. As DU-145 cells were exposed to medium containing decreasing concentrations of glucose, they exhibited a dose-responsive increase in sensitivity to killing by TRAIL (Fig. 1).

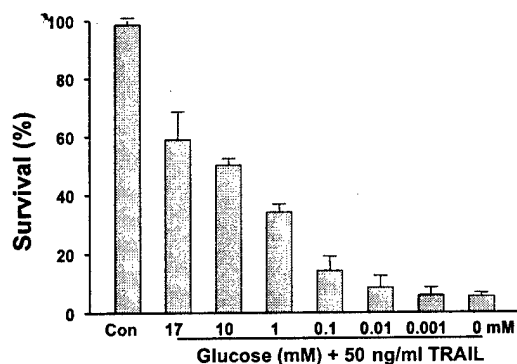


Figure 1. Effect of various concentrations of glucose on TRAIL-induced cytotoxicity in DU-145 cells.

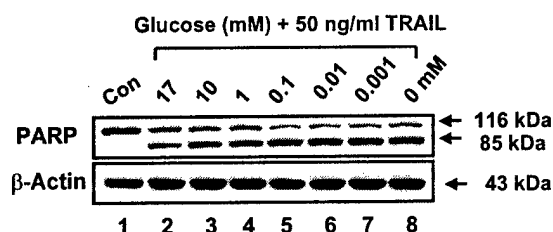


Figure 2. Effect of various concentrations of glucose on TRAIL-induced proteolytic cleavage of PARP. DU-145 cells were treated with 50 ng/ml TRAIL for 4 hr in the presence of various concentrations of glucose (0-17 mM) and then harvested. Lysates from equal amounts of protein (20 μ g) were separated by SDS-PAGE and immunoblotted. The upper band indicates 116 kDa PARP whereas the lower band indicates the 85 kDa apoptosis-related cleavage fragment. **Con**, untreated control cells. **Actin**, actin was used to confirm that similar amounts of protein were loaded in each lane.

Additional studies were designed to determine whether the combination of TRAIL and glucose deprivation enhances poly (ADP-ribose) polymerase (PARP) cleavage, a hallmark feature of apoptosis (Fig. 2). Glucose deprivation alone failed to induce PARP cleavage (data not shown). In contrast, TRAIL (50 ng/ml) alone caused PARP cleavage. PARP (116 kDa) was cleaved into characteristic 85 kDa fragments in the presence of TRAIL. PARP cleavage was markedly enhanced when TRAIL and low glucose were combined; cleavage increased as the glucose concentration decreased (Fig. 2). These results show that low glucose significantly enhances TRAIL-induced apoptosis. Thus, these experimental data support

the clinical significance of our studies, by identifying a specific signal transduction pathway with the potential to improve the efficacy of cancer therapy.

For the application of glucose oxidase to TRAIL-based gene therapy, we took advantage of hallmark features of the tumor microenvironment to develop a novel gene therapy. Previous studies demonstrate that tumors generally have lower concentrations of glucose than normal tissue. Generally, glucose concentrations of the serum and the interstitial fluid in Walker 256 mammary carcinoma are 9.5 mM and 0.03 mM, respectively. These data suggest that the extracellular space (vascular and interstitial compartments) of solid tumors contains variable concentrations of glucose. The level of glucose probably depends on the proximity of the vascular space to the tumor cells. The clinical efficacy of TRAIL may be closely tied to the glucose concentration in the space surrounding each tumor cell. In the near future, we will develop a TRAIL-based gene therapy in conjunction with immunotherapy. Currently we are collaborating with Dr. R.V. Blackburn at ApoLife, Inc for this project. We will employ a glucose oxidase (GOD) immunotoxin to further deplete glucose in the tumor. Moreover, GOD generates hydrogen peroxide. Our preliminary data demonstrate that TRAIL cytotoxicity is potentiated in the presence of GOD (Fig. 3). The combination of TRAIL and GOD effectively increased cell death compared to cells treated with TRAIL alone or GOD alone.

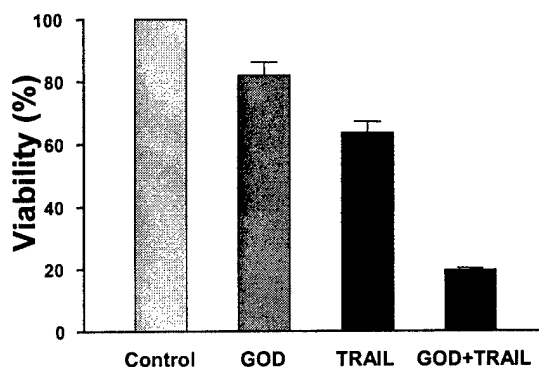


Figure 3. Effect of GOD on TRAIL-induced cytotoxicity in DU-145 cells. Cells were treated with 50 mU/ml GOD alone, 50 ng/ml TRAIL alone, or GOD + TRAIL for 3 hr. Survival was determined by the Trypan exclusion assay.

This idea is based on previous observations that under physiological conditions, GOD catalyses the oxidation of β -D-glucose to D-glucose-1,5-lactone, which is subsequently hydrolysed to gluconic acid. The reaction also results in the production of hydrogen peroxide. Hydrogen peroxide exerts its effect by formation of hydroxyl radicals, which can damage various cellular components, causing DNA strand breaks, protein modification, and lipid peroxidation (Vallyathan and Shi, 1997). *In vitro* and *in vivo* studies have demonstrated that GOD has potent tumoricidal activity (Higuchi et al., 1991; Nathan and Cohn, 1981; O'Donnell-Tormey et al., 1985; Samoszuk, M.K. et al., 1989). Hydrogen peroxide produced by GOD is effective in preventing tumor growth (Higuchi et al., 1991), and its effect can be enhanced by hydrogen peroxide decomposition inhibitors such as 3-aminotriazole, hydroxylamine and sodium azide (Higuchi et al., 1991). Moreover, by comparison to normal cells, tumor cells are more susceptible to GOD (Mavrier, P. et al., 1988; Combs et al., 1993). Figure 4 demonstrates the cytotoxic effect of GOD in DU-145 cells exposed to various concentrations of purified GOD proteins for 3 hr or 4 hr. Survival was determined by the Trypan blue exclusion assay.

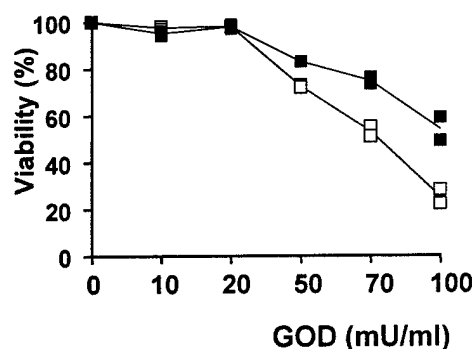


Figure 4. DU-145 cell survival after treatment with various concentrations of GOD (10-100 mU/ml) for 3 hr (■) or 4 hr (□).

Figure 5 clearly shows that TRAIL-induced activation of caspase-3,-8,-or 9 was promoted by treatment with GOD. GOD (50 mU/ml) alone did not induced the activation of caspases (lane 2 in Fig. 5).

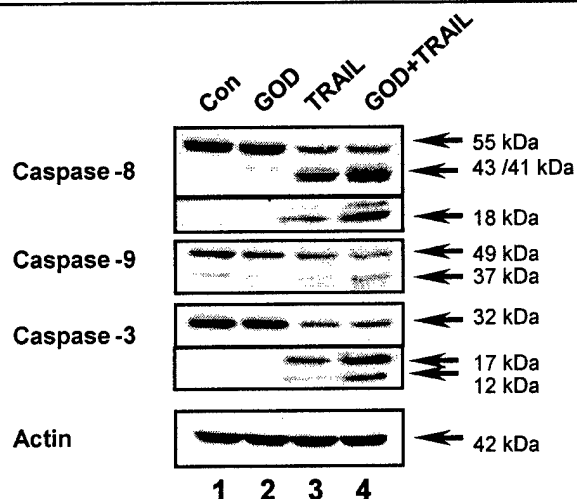


Figure 5. Effect of GOD on TRAIL-induced activation of caspases in DU-145 cells. Cells were treated with 50 mU/ml GOD alone, TRAIL (50 ng/ml) alone, or GOD + TRAIL for 3 hr and then harvested. Lysates containing equal amounts of protein (20 μ g/ml) were separated by SDS-PAGE and immunoblotted. Con, untreated control cells.

Key research accomplishments

- 1) Clinically relevant concentrations (0.01-0.1 mM) of glucose promoted TRAIL-induced cytotoxicity.
- 2) Glucose oxidase enhances TRAIL-induced cytotoxicity. We are currently building on this observation by developing a novel TRAIL-based gene therapy in conjunction with GOD immunotoxin.

Reportable Outcomes

Entitled "Reconstitution of galectin-3 alters glutathione content and potentiates TRAIL-induced cytotoxicity by dephosphorylation of Akt" (2003) has been accepted in Experimental cell Research, in press.

Conclusions

Glucose oxidase enhances TRAIL-induced cytotoxicity *in vitro*. Therefore, it can be useful to

combine the effect of TRAIL and GOD in the form of plasmid instead of using the adenovirus as a vector system.

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Revised STATEMENT OF WORK

Task 1: Demonstration of the enhanced *in vitro* cytotoxicity on prostate tumor cells by combining TRAIL and lowered glucose was assessed (1-6 months).

Task 2: Demonstration of the enhanced *in vitro* cytotoxicity on prostate tumor cells by combining TRAIL and GOD was assessed (7-12 months).

Task 3: Examination of tumoricidal effect of plasmid expressing TRAIL on human prostate tumor xenografts in mice (13-18 months).

- a) Construction of hFlex/TRAIL recombinant plasmid (13-14)
- b) Tumoricidal effect of TRAIL by using hydroxydynamic-based gene delivery protocol (15-16)

Task 4: Tumoricidal effect of GOD either alone or in combination with TRAIL will be assessed by the tumor growth and animal survival assay in mice bearing the human prostate cancer xenograft (17-24)

- a) Construction of recombinant GOD immunotoxin and its tumoricidal effect on human tumors growing in nude mice (17-20).
- b) Tumoricidal effect in combination with TRAIL and GOD plasmids will be assessed *in vitro* as well as *in vivo* (21-24).

Reconstitution of Galectin-3 alters Glutathione Content and Potentiates TRAIL-Induced Cytotoxicity by Dephosphorylation of Akt

Yong J. Lee^{1*}, Young K. Song¹, Jae J. Song¹, R. Rita Siervo-Sassi², Hyeong-Reh C. Kim³, Ling Li⁴, Douglas R. Spitz⁴, Anna Lokshin², and Jin H. Kim¹

¹Department of Surgery, University of Pittsburgh, Pittsburgh, PA 15213, USA; ²Magee Women's Research Institute, Pittsburgh, PA 15213, USA; ³Department of Pathology, Wayne State University School of Medicine, Detroit, Michigan, MI 48201, USA; ⁴Free Radical and Radiation Biology Program, Department of Radiation Oncology, The University of Iowa, Iowa City, Iowa 52242

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*All Correspondence to:

Dr. Yong J. Lee
University of Pittsburgh, Department of Surgery, The Hillman Cancer Center, 5117 Centre Ave.,
Room G.5a, Pittsburgh, PA 15213, U.S.A.
Tel +82-412-623-3268
Fax +82-412-623-1010
E-mail:leeyj@msx.upmc.edu

Abbreviations: DcR1, decoy receptor 1; DcR2, decoy receptor 2; DR4, death receptor 4; DR5, death receptor 5; FADD, Fas-associated death domain; FasL, Fas ligand; FLICE, Fas-associated death domain-like interleukin-1 β -converting enzyme; FLIP, FLICE inhibitory protein; PBS, phosphate-buffered saline solution; SDS, sodium dodecyl sulfate; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; PARP, poly (ADP-ribose) polymerase; ROS, reactive oxygen species; TNF, tumor necrosis factor; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand ; GSH, glutathione; GSSG, glutathione disulfide; PI, propidium iodide; FITC, fluorescein isothiocyanate

Abstract

We investigated the role of galectin-3 in tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptotic death in human breast carcinoma BT549 cells. We observed that parental galectin-3 null BT549 cells (BT549_{par}) as well as control vector transfected (BT549_{neo}) cells were resistant to TRAIL, while galectin-3 cDNA transfected BT549 cells (BT549_{gal-3}) were sensitive to TRAIL. Data from flow cytometry and immunoblotting analyses reveal that reconstitution of galectin-3 promoted cell death and PARP cleavage as well as caspase (-8, -9, - and -3) activation during TRAIL treatment. However, unlike TRAIL treatment, galectin-3 transfectants were resistant to UV-B-induced PARP cleavage. Data from cDNA array analysis show that galectin-3 did not significantly enhance or reduce any apoptosis-related gene expression. Moreover, although galectin-3 restored pre-mRNA splicing activity and resulted in elevation of FLIPs protein, experiments with FLIPs cDNA transfected cells show that overexpression of FLIPs did not sensitize cells to TRAIL. Interestingly, BT549_{gal-3} cells demonstrated a ~2-fold increase in total glutathione content as well as a ~5-fold increase in GSSG content in comparison to BT549_{par} and BT549_{neo} cells suggesting that galectin-3 overexpression may alter intracellular oxidation/reduction reactions affecting the metabolism of glutathione and other thiols. In addition, galectin-3 overexpression inactivated Akt by dephosphorylation. Finally, overexpression of constitutively activated Akt protected BT549_{gal-3} cells from TRAIL-induced cytotoxicity. Taken together, our data suggest that galectin-3-enhanced TRAIL-induced cytotoxicity is mediated through dephosphorylation of Akt possibly through a redox dependent process.

Key Words: Galectin-3, TRAIL, Apoptosis, cDNA array, FLIPs, Akt

Introduction

Galectin-3 (also known as Mac-2, CBP-35, IgEBP, CBP-30, RL-29, L-29, hL-31, and LBL) is a M_r 31 kDa carbohydrate-binding protein with affinity for beta-galactosides [1]. It is composed of three distinct domains: an NH₂-terminal of 12 amino acids with a leader sequence containing a casein kinase I serine phosphorylation site, which is preceded by a collagenase-sensitive Pro-Gly-rich motif, and a globular COOH-terminal domain containing the carbohydrate-binding site [2,3]. The protein plays a role in cell-cell and cell-matrix interactions [4,5], induction of pre-mRNA splicing [6], cell proliferation [7], cell cycle regulation [8], and angiogenesis [9]. It is found at elevated levels in a wide variety of neoplastic cells and thought to be involved in cognitive cellular interactions during transformation and metastasis [10]. Human breast carcinoma BT549 cell line has been widely used to investigate the function of galectin-3 since it does not express this protein. Introduction of human galectin-3 cDNA into BT549 cells, which is non-tumorigenic in nude mice, resulted in upregulation of the L1 retrotransposon. This molecule may play a role in the tumorigenicity of some breast cancers [11]. Several studies also demonstrated that galectin-3 has antiapoptotic activity [5,12,13]. It protects against apoptotic cell death in response to a variety of apoptosis inducing stimuli including staurosporine, anti-Fas antibody, cisplatin, tumor necrosis factor- α , menadione, and loss of cell adhesion [5,8,12,13,14]. It also has been suggested to protect cells from oxidative stress-induced cell death [15,16].

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)/APO-2L is a type II integral membrane protein belonging to the tumor necrosis factor (TNF) family. TRAIL is a 281-amino acid protein, related most closely to Fas/APO-1 ligand. Like Fas ligand (FasL) and TNF, the C-terminal extracellular region of TRAIL (amino acids 114-281) exhibits a homotrimeric subunit structure [17]. The apoptotic signal induced by TRAIL is transduced by its binding to the death receptors TRAIL-R1 (DR4) and TRAIL-R2 (DR5), which are members of the TNF receptor superfamily. Both DR4 and DR5 contain a cytoplasmic death domain that is required for TRAIL receptor-induced apoptosis. Recent studies demonstrated that the Fas-associated death

domain (FADD) is required for TRAIL-induced apoptosis [18,19,20,21]. TRAIL triggers apoptosis by recruiting the apoptosis initiator procaspase-8 through the adaptor FADD [18,22]. Caspase-8 can directly activate downstream effector caspases including procaspase-3, -6, and -7 [23]. Caspase-8 also cleaves Bid and triggers mitochondrial damage that in turn leads to cytochrome c release [24,25]. Cytochrome c in the cytoplasm binds to Apaf-1, which then permits recruitment of procaspase-9. Caspase-9 cleaves and activates procaspase-3 [26]. In addition, TRAIL-induced mitochondrial damage may cause cellular production of reactive oxygen species (ROS) which leads to cell death. Previous studies have revealed that TNF treatment causes ultrastructural abnormalities of mitochondria [27,28] and elevates ROS production through the mitochondrial respiratory chain [28,29].

In this study, we hypothesized that galectin-3 might protect cells from TRAIL-induced cytotoxicity, perhaps by inhibiting oxidative stress. Unexpectedly, we observed that galectin-3-transfected BT549 (BT549_{gal-3}) cells were sensitive to TRAIL-induced cytotoxicity, but this did not appear to be related to changes in apoptosis-related gene expression or anti-apoptotic c-FLIP pre-mRNA splicing. Interestingly, the galectin-3-transfected BT549 cells demonstrated an ~2-fold increase in total glutathione content as well as an ~5-fold increase in glutathione disulfide, relative to vector controls. These results suggest that galectin-3 overexpression may alter intracellular metabolic oxidation/reduction reactions involving glutathione and other thiols which could play an important role in determining the intrinsic sensitivity to TRAIL. In addition, our results showed that dephosphorylation of the antiapoptotic signaling molecule, Akt, in BT549_{gal-3} is strongly associated with an increase in sensitivity to TRAIL.

Materials and Methods

Cell culture and survival determination

The human breast carcinoma BT549 (BT549_{par}), its stable clones expressing the human galectin-3 proteins (BT549_{gal-3}, clone #11914, #11811, and #11913), and its control clone (BT549_{neo}) were obtained from Dr. A. Raz (Wayne State University, Detroit, MI, USA). BT549_{par} has a galectin-3 null phenotype and shows to be nontumorigenic in athymic nude mice. All cell lines were grown in Dulbecco's modified Eagle medium/nutrient mixture F-12 (DMEM/F-12, Life Technologies, Rockville, MD, USA) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT, USA), and 2 mM L-glutamine in a 95% air and 5% CO₂ incubator at 37°C. For Trypan blue exclusion assay [30], trypsinized cells were pelleted and resuspended in 0.2 ml of medium, 0.5 ml of 0.4% Trypan blue solution and 0.3 ml of phosphate-buffered saline solution (PBS). The samples were mixed thoroughly, incubated at room temperature for 15 min and examined.

Reagents and Antibodies

Purification of His-tagged human TRAIL fragment (amino acids 114-281) was previously described [31]. Polyclonal anti-caspase-3 and anti-caspase-9 antibodies were purchased from Santa Cruz (Santa Cruz, CA, USA). Anti-DR5 and anti-DcR2 from StressGen (Victoria, BC, Canada), anti-DR4 from Upstate Biotechnology (Lake Placid, NY, USA), anti-FLIP from Calbiochem (San Diego, CA, USA), anti-clAP-2 from R&D systems (Minneapolis, MN, USA) and anti-phospho-Ser-473 Akt and anti-Akt from New England Biolabs (Beverly, MA, USA) were used. Anti-galectin-3 antibody was obtained from Dr. H.R.C. Kim (Wayne State University, Detroit, MI, USA). Monoclonal antibodies were purchased from the following companies: anti-caspase-8 and anti-FADD from Upstate Biotechnology (Lake Placid, NY, USA), anti-DAP3 from BD Bioscience (Franklin Lakes, NJ, USA), anti-Bcl-2 from ICN (Costa Mesa, CA, USA) and anti-

PARP from Biomol Research Laboratory (Plymouth Meeting, PA, USA). Other chemicals were purchased from Sigma (St. Louis, MO, USA).

Morphological evaluation

Approximately 5×10^5 cells were plated into 60-mm dishes overnight. Cells were treated with TRAIL and then analyzed by phase contrast microscopy for signs of apoptosis.

Protein extracts and polyacrylamide gel electrophoresis (PAGE)

Cells were lysed with 1 x Laemmli lysis buffer (2.4 M glycerol, 0.14 M Tris, pH 6.8, 0.21 M sodium dodecyl sulfate, 0.3 mM bromophenol blue) and boiled for 10 min. Protein content was measured with BCA Protein Assay Reagent (Pierce, Rockford, IL, USA). The samples were diluted with 1x lysis buffer containing 1.28 M β -mercaptoethanol, and equal amount of protein was loaded on 8-12 % sodium dodecyl sulfate (SDS)-polyacrylamide gels. SDS-PAGE analysis was performed according to Laemmli [32] using a Hoefer gel apparatus.

Immunoblot analysis

Proteins were separated by SDS-PAGE and electrophoretically transferred to PVDF membrane. The membrane was blocked with 7.5 % nonfat dry milk in PBS-Tween-20 (0.1 %, v/v) at 4 °C overnight. The membrane was incubated with primary antibody (diluted according to the manufacturer's instructions) for 1 hr. Horseradish peroxidase conjugated anti-rabbit or anti-mouse IgG was used as the secondary antibody. Immunoreactive protein was visualized by the chemiluminescence protocol (ECL, Amersham, Arlington Heights, IL, USA).

Assay of caspase activity

Caspase activity was measured using fluorogenic caspase substrates as previously described. [31]. Briefly, cells were harvested after incubation with TRAIL and then lysed in caspase lysis buffer (0.03% Nonidet, 1 mM DTT, 50 mM Tris, pH 7.5). The insoluble fractions were removed by centrifugation for 5 min (800g), and the cytosolic fractions were incubated with caspase reaction buffer (50 mM NaCl, and 2.5 mM DTT, 20 mM HEPES, pH 7.5), containing 10

mM DEVD-amc for caspase-3-like activity, Ac-IETD-afc for caspase-8-like activity, or Ac-LEHD-afc for caspase-9-like activity, in a total volume of 200 μ l for 100 min at 37°C. Fluorescence, released by caspase activity, was measured using 360 nm excitation for amc or 400 nm for afc. The intensity at the optimum (450 nm for amc or 505 nm for afc) was measured using a Spectra Max Gemini Plate Reader (Molecular Devices, Sunnyvale, CA, USA).

Flow Cytometry.

Cells were pelleted and washed with FACS buffer (phosphate-buffered saline, PBS, 1% BSA, 0.1% sodium azide). Apoptosis was evaluated using annexin V binding assay (Beckman Coulter, Brea, CA) according to the manufacturer's instructions. Cells were double-stained with FITC-conjugated annexin V and propidium iodide (PI) for 20 min at 4°C. Analysis was performed using the FACScan flow cytometer, and results were analyzed with CellQuest software (both from Becton Dickinson Immunocytometry Systems, San Jose, CA, USA).

UV-irradiation

Cells were plated in 60-mm plates and exposed to UV radiation using a Spectrolinker (Spectronics Corp., Westbury, NY, USA).

cDNA array hybridization and analysis

BT549neo and BT549gal-3 cells were suspended in TRIzol reagent (Gibco BRL, Grand Island, NY, USA) according to the manufacturer's procedures. Poly(A)⁺ RNA was purified using Oligotex messenger RNA (mRNA) isolation kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. The cDNA probes were synthesized from 1 μ g mRNA by reverse transcription using 35 μ Ci (1.295 KBq) [³²P]dATP (3.000 Ci/mmol; 111 TBq/mmol) (NEN, Boston, MA, USA). Reverse transcribed products were purified using NICK Columns (Amersham Pharmacia Biotech, Uppsala, Sweden). Human Apoptosis Expression Array membranes containing 219 cDNAs (R&D Systems, Minneapolis, MN, USA) were prehybridized and hybridized using High Efficiency Hybridization System (Molecular Research Center,

Cincinnati, OH, USA) according to manufacturer's instructions. The denatured probes were added directly into the hybridization buffer and incubated for 18 hr at 60°C. The membranes were washed 4 times for 30 minutes at 68°C with Washing and Pre-Hyb Solution (Molecular Research Center). Triplicate measurements were analyzed for reproducibility. The membranes were exposed to Kodak film at -70°C for 6 hr. The hybridization signals were photometrically evaluated using ArrayVision Software (Imaging Research, St. Catharines, ON, Canada) and normalized to the expression of β -actin, β_2 -macroglobulin and α -tubulin. For a given gene, positive ratio corresponded to the normalized value of the intensity of a gene in treated cells divided by the normalized value in untreated cells. Ratios greater than or equal to 2 and less than or equal to 2 were identified for further investigation. All the expressed genes that did not meet these criteria can be found in R&D Handbook (Cat No. GA002).

Measurement of glutathione

Cells were washed with ice cold phosphate-buffered saline (PBS), scraped into cold PBS, and centrifuged at 4°C for 5 min at 400 x *g* to obtain cell pellets, which were then frozen at -80°C. Pellets were then thawed and homogenized in 50 mM potassium phosphate buffer pH 7.8 containing 1.34 mM diethylenetriaminepentaacetic acid. Total glutathione content was determined by the method of Anderson [33]. Reduced and oxidized glutathione were distinguished by addition of 2 μ l of a 1:1 mixture of 2-vinylpyridine and ethanol per 30 μ l of sample followed by incubation for 1 hr and assay as previously described by Griffith [34]. All glutathione determinations were normalized to the protein content of whole homogenates using the method of Lowry et al. [35].

Transfection or infection

For transfection, BT549 cells were transfected with pcDNA3-Flag-FLIPs (a gift from Dr. P.H. Krammer, German Cancer Research Center, Heidelberg, Germany) using LipofectAMINE Plus (Gibco BRL Life Technologies, Grand Island, NY, USA). Transfected cells were selected for 3

weeks in growth medium containing 0.4 mg of G418 (Geneticin: Gibco BRL Life Technologies) per ml. For infection, BT549 cells were infected with adenoviral vectors containing constitutively activated Akt (myr-Akt: a gift from Dr. N.N. Ahmed and P. Tsichlis, Fox Chase Cancer Center, Philadelphia, PA, USA) or wild-type Akt (wt-Akt). Infections with various multiplicities of infection (MOIs) of recombinant adenovirus were performed in DMEM media supplemented with 10% fetal bovine serum for 24 hrs.

Results

Expression of galectin-3 promotes TRAIL-induced cytotoxicity

We previously observed that introduction of human galectin-3 cDNA into BT549 cells results in acquisition of a resistance to oxidative stress [15,16]. To examine whether galectin-3 transfected BT549 cells (Fig. 1A) are resistant to TRAIL, cells were treated with 100 ng/ml TRAIL for 2 hr. Figure 1B, and 1C show that little or no cytotoxicity was observed with TRAIL in parental BT549 (BT549_{par}) and control vector transfected BT549 (BT549_{neo}) cells. In contrast, galectin-3 transfected BT549 cells (clone #11914, #11811, and #11913) were sensitive to TRAIL. TRAIL-induced cell death was concentration dependent (Fig. 1D). Interestingly, the survival curve became biphasic as the TRAIL dose was increased. This is probably due to the saturation of TRAIL cytotoxic effect at 100 ng/ml or the TRAIL resistant population.

To determine whether TRAIL-induced cytotoxicity resulted from necrotic death or apoptotic death, we examined the mode of cell death following TRAIL treatment. BT549_{par}, BT549_{neo}, or BT549_{gal-3#11914} cells were treated with 100 ng/ml TRAIL for 4 hr, and then stained with annexin V and propidium iodide (PI), followed by flow cytometric analysis. The appearance of phosphatidylserine on the cell surface, recognized as a universal feature of apoptosis, was detected by annexin V-FITC (fluorescein isothiocyanate) staining, while necrotic cells were detected by PI staining. Figures 2A and 2B show that TRAIL did not induce cell death in

BT549_{neo} cells. Similar results were observed in BT549_{par} (data not shown). Unlike BT549_{neo} cells, TRAIL effectively induced necrotic cell death as well as apoptotic cell death in BT549_{gal-3#11914} cells (Fig. 2C vs. Fig. 2D).

Reconstitution of galectin-3 potentiates TRAIL-induced caspase activation and PARP cleavage

It is well known that TRAIL-induced apoptotic death is mediated through a caspase cascade. To examine whether galectin-3 enhances TRAIL-induced caspase activation, several caspases (caspase-8, -9 and -3) were examined. Western blot analysis shows that procaspase-8 (54/55 kDa), procaspase-9 (46 kDa), and procaspase-3 (32 kDa) were cleaved to the intermediates (41/43 kDa, 37 kDa, and 20 kDa) and active form (17 kDa) in the presence of 100 ng/ml TRAIL in BT549_{gal-3} cells, while TRAIL did not activate caspases in BT549_{par} and BT549_{neo} cells (Fig. 3A). Similar results were observed in an enzyme activity assay (Fig. 3B). These three caspases were activated within 45 min during 100 ng/ml TRAIL treatment. As shown in Figure 3A, the activation of caspases was also dependent upon the dose of TRAIL in BT549_{gal-3} cells. We extended our studies to investigate caspase-mediated cleavage of poly(ADP-ribose) polymerase (PARP). PARP (116 kDa) was cleaved yielding a characteristic 85 kDa fragment in the presence of 100 ng/ml TRAIL in BT549_{gal-3} cells (Fig. 3A) but not BT549_{par} and BT549_{neo} cells (Fig. 3A).

Reconstitution of galectin-3 suppresses UV-induced PARP cleavage

Previous studies have shown that galectin-3 protects cells from apoptotic agents including cisplatin and staurosporine [12, 14]. In contrast, we observed that galectin-3 promotes TRAIL-induced cytotoxicity (Figs. 1 and 2). As a control, an additional pro-apoptotic stimulus, e.g. UV radiation, was considered in order to demonstrate the specificity of the results obtained. Cells (BT549_{par}, BT549_{neo}, and BT549_{gal-3#11914}) were exposed to 3000 joules/m² of UV-B radiation, PARP cleavage, a hallmark feature of apoptosis, was analyzed various times (4-12 hr) after UV-irradiation. Figure 4 shows that, in contrast to the results with TRAIL, UV-B-induced PARP

cleavage in BT549_{par} and BT549_{neo} cells, but not BT549_{gal-3#11914} cells. These results provide evidence that overexpression of galectin-3 in a cellular background (BT549) where sensitization to TRAIL-induced PARP cleavage was seen, also resulted in protection against UV-induced PARP cleavage.

Effect of galectin-3 on apoptosis-related gene expression

To investigate whether reconstitution of galectin-3 alters apoptosis-related gene expression in BT549 cells, Human Apoptosis Expression Array membranes containing 219 cDNAs were employed. Data from the expression array show that there was no significant increase or decrease in the apoptosis-related gene expression (Fig. 5). Western blot analysis in Figure 6 also shows little or no difference in the intracellular level of death receptors (DR4, DR5), decoy receptor (DcR2), death associated proteins (DAP3, FADD), and antiapoptotic proteins (Bcl-2).

Effect of galectin-3 on the intracellular level of glutathione

Previous studies suggest that TRAIL-induced cytotoxicity is mediated through ROS production [28]. We hypothesized that galectin-3 may enhance TRAIL-induced cytotoxicity by reducing the levels of intracellular thiol antioxidants. To test our hypothesis, the intracellular levels of total glutathione and glutathione disulfide were measured in BT549_{par}, BT549_{neo}, and BT549_{gal-3#11914} cells. Figure 7 shows that BT549_{gal-3#11914} cells demonstrate a 2-fold increase in total glutathione content as well as a 5-fold increase in glutathione disulfide content in comparison to BT549_{par} and BT549_{neo} cells. These results suggest that galectin-3 over expression may alter intracellular metabolic oxidation/reduction reactions resulting in an increased demand for glutathione synthesis as well as an increase in GSSG content that could play an important role in determining the intrinsic sensitivity to TRAIL.

Effect of galectin-3 on c-FLIP mRNA splicing

Previous studies have shown that galectin-3 is involved in pre-mRNA splicing [6]. We examined whether reconstitution of galectin-3 restores splicing activity in BT549_{gal-3#11914} cells.

Data from Figure 8 show that the intracellular level of c-FLIPs was increased in BT549_{gal-3#11914} cells. This is probably due to splice variants of c-FLIP mRNA [36]. These variant forms have differential functions [37]. Interestingly, c-FLIP has been controversially reported to possess apoptosis-promoting [7,38, 39] and -inhibiting functions [36, 37, 40]. We further investigated whether elevation of c-FLIPs is responsible for enhancement of TRAIL-induced apoptotic death in BT549_{gal-3#11914} cells. BT549_{par} cells were stably transfected with the plasmid pcDNA3-Flag-FLIPs containing Flag-tagged FLIPs cDNA (Fig. 9A). Three transfectants (#7, #11, and #12) were chosen for further experiments. Figure 9B and 9C show that expression of FLIPs neither potentiated TRAIL-induced cytotoxicity nor enhanced PARP cleavage.

Effect of galectin-3 on Akt phosphorylation

Recent studies have shown that dephosphorylation of Akt sensitizes TRAIL-induced cytotoxicity [41, 42, 43]. We examined whether expression of galectin-3 results in dephosphorylation of Akt and subsequently BT549_{gal-3} cells become sensitive to TRAIL. Figure 10 shows that serine 473 residue in Akt is dephosphorylated in galectin-3 cDNA stably transfected BT₅₄₉ cells. These results were observed in three different BT549_{gal-3} clones (#11914, #11811, and #11913). To test whether dephosphorylation of Akt is responsible for promoting sensitivity to TRAIL, BT549_{gal-3#11914} cells were infected with adenoviral vectors containing a constitutively activated Akt (Ad.myr-Akt) or wild-type Akt (Ad.wt-Akt). Figure 11A shows that the level of Akt was increased by infection with these adenoviral vectors. The level of Akt was dependent upon MOIs. Figure 11A also shows that an increase in phosphorylated Akt (an active form of Akt) was observed in Ad.myr-Akt transfected cells, but not Ad.wt-Akt transfected cells. These results suggest that galectin-3 dephosphorylated wild-type Akt in BT549_{gal-3#11914} cells. Overexpression of myr-Akt, but not wt-Akt, enhanced resistance to TRAIL (Fig. 11B). For example, when BT549_{gal-3#11914} cells were infected with Ad.myr-Akt at an MOI of 100, prior to treatment with 100 ng/ml TRAIL for 2 hr, survival was enhanced by approximately

5-fold. Taken together, the reconstitution of galectin-3 dephosphorylates Akt and the dephosphorylation of Akt may cause the enhanced sensitivity to TRAIL.

Discussion

Previous studies have shown that there are two signaling pathways that lead to apoptotic cell death. One is the extrinsic pathway and the other is the intrinsic pathway. The extrinsic pathway is mediated through death receptors while the intrinsic pathway is mediated by mitochondria. TRAIL, which binds to the death receptors (DR4 and DR5), activates caspase-8 and subsequently activates downstream effectors such as caspase-3 [31]. Recently, we have observed that TRAIL-induced apoptosis is primarily dependent upon a mitochondria-independent pathway [31]. TRAIL-induced apoptosis is not significantly inhibited by Bcl-2 overexpression [44, 45]. However, a number of reports show that Bcl-2 can significantly inhibit TRAIL cytotoxicity and caspase-3 activation by inhibiting cytochrome *c* release from mitochondria [46, 47, 48]. This discrepancy is probably due to the type of cell responding to the TRAIL-signal.

Previous studies have shown that several c-FLIP splice variants exist on the mRNA level, but two endogenous forms, c-FLIP_L and c-FLIPs are detected on the protein level [49, 50]. Our data from Figure 8 indicate that galectin-3 is involved in the splicing of pre-mRNA and produces c-FLIPs. The role of c-FLIP in apoptosis signaling has been controversial. Some reports have described c-FLIP as an antiapoptotic molecule [36,38,40] and others as a proapoptotic molecule [7,38,39]. Our studies reveal that FLIPs has an antiapoptotic function (Fig. 9). Recent studies show that FLIP_L and FLIPs prevent caspase-8 activation at different levels of procaspase-8 processing at the death-inducing signaling complex (DISC). FLIPs completely inhibits cleavage of procaspase-8, whereas FLIP_L inhibits the second cleavage step of procaspase-8 [37]. Nonetheless, our studies show that in spite of increasing the level of FLIPs, galectin-3

transfected BT549_{gal-3} cells are sensitive to TRAIL. These results suggest that FLIPs is not responsible for galectin-3 promoted TRAIL-induced apoptotic death.

We have observed that galectin-3 expression in our system protects cells from UV-induced PARP cleavage (Fig. 4), anoikis [8], and oxidative stress-induced cell death [15,16]. Interestingly, data from micrographs showed that cell detachment only occurred in BT549_{gal-3} cells during TRAIL treatment (Fig. 1B). Moreover, Figure 7 shows that the intracellular level of glutathione, an important cofactor for the enzymatic decomposition of intracellular peroxides by the glutathione peroxidase enzyme, is approximately 2-fold higher in BT549_{gal-3} cells, relative to BT549_{par} and BT549_{neo} cells, suggesting an increased demand for glutathione synthesis. In addition, the oxidized form of glutathione (GSSG) also increased approximately 5-fold and the ratio of GSH/GSSG decreased from approximately 20:1 to 8:1 in the galectin-3 overexpressing cells. These results suggest that overexpression of galectin-3 may have caused a shift in oxidative metabolism resulting in an increased demand for glutathione synthesis as well as more oxidizing intracellular environment. This kind of shift in oxidative metabolism may have contributed to the sensitization of the cells to TRAIL-induced cytotoxicity as well as induction of an adaptive response that could have rendered cells resistant to other forms of oxidative stress.

Yang et al. [14] reported that galectin-3 contains a four amino acid motif (NWGR) conserved in the BH1 domain of the Bcl-2 gene family. The NWGR motif is responsible for the antiapoptotic activity of Bcl-2 [14]. A change of glycine to alanine in this motif abrogates its antiapoptotic activity [12]. Overexpression of galectin-3 probably protects cells from apoptotic death by inhibiting the caspase pathway [12] or by maintaining mitochondrial homeostasis [13]. However, our data clearly show that galectin-3 transfected BT549 cells become sensitive to TRAIL-induced apoptotic death. A fundamental question which remains unanswered is how galectin-3 can induce both protective as well as sensitizing effects. One possibility is that reconstitution of galectin-3 regulates the extrinsic pathway-associated gene expression. Recent studies have shown that genotoxic agents such as chemotherapeutic agents and ionizing

radiation sensitize to TRAIL-induced cytotoxicity by decreasing intracellular levels of FLIP or increasing DR5 gene expression [51, 52]. However, unlike genotoxic agents, the reconstitution of galectin-3 does not down-regulate FLIP or up-regulate DR5 gene expression (Figs. 6 and 8) in BT549 cells. At the present time, we can only speculate how the reconstitution of galectin-3 sensitizes to TRAIL-induced cytotoxicity. Our studies reveal that expression of galectin-3 dephosphorylates Akt in BT549_{gal-3} cells (Fig. 11). Recent studies reveal that Akt phosphorylates Bad, a proapoptotic member of the Bcl-2 family [53, 54]. Phosphorylated Bad is no longer capable of forming heterodimers with Bcl-X_L or Bcl-2 [55, 56], and associates with 14-3-3 proteins. Association of Bad with 14-3-3 proteins may protect Bad from dephosphorylation or sequester Bad away from its targets at the mitochondria. In contrast, dephosphorylation of Bad results in targeting of Bad to mitochondrial membranes where Bad has been proposed to interact with and inactivate Bcl-X_L, an antiapoptotic protein. Akt also phosphorylates procaspase-9 and inhibits its protease activity [57]. Thus, dephosphorylated Akt may no longer phosphorylate Bad or procaspase-9. This possibility needs to be investigated further.

At this time only speculation can be made concerning the mechanism of Akt dephosphorylation in BT549_{gal-3} cells. Recent studies have revealed that elevation of intracellular ceramide levels inhibits either phosphatidylinositol 3-kinase [PI(3)K] [58] and/or activates ceramide-activated protein phosphatase (CAPP) [59]. PI(3)K phosphorylates phosphoinositides at the 3'-position of the inositol ring, and its major lipid product is phosphatidylinositol 3,4,5-triphosphate (PIP₃) [60]. PIP₃ facilitates the recruitment of Akt to the plasma membrane through binding with the pleckstrin homology (PH) domain of Akt [60]. Akt is activated by phosphoinositide-dependent kinase-1 (PDK1) through phosphorylation at threonine 308 and serine 473 [61, 62]. The inhibition of PI(3)K results in preventing the translocation of Akt to the plasma membrane. Unlike PI(3)K, CAPP, which is a member of the 2A class of Ser/Thr protein phosphatases (PP2A) [63, 64], inhibits Akt by promoting dephosphorylation of serine 473 [65]. We postulate that reconstitution of galectin-3 elevates the intracellular level of

ceramide and subsequently inactivates PI(3)K or activates CAPP. The inactivated PI(3)K and/or activated CAPP could then potentially lead to the dephosphorylation Akt and consequently enhance sensitivity to TRAIL. Since Akt as well as PI(3)K and CAPP have also been suggested be regulated by intracellular oxidation reduction reactions (66-68), these proposed pathways may also be related to the galectin-3 mediated alterations in glutathione metabolism that were observed. Overall, our proposed model may provide important insights into the understanding of how reconstitution of galectin-3 leads to dephosphorylation of Akt. This model may also provide a framework for studying the critical steps in the dephosphorylation of Akt in BT549_{gal-3} cells.

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Figure Legends

Figure 1. Reconstitution of galectin-3 promotes TRAIL-induced cytotoxicity in human breast carcinoma BT549 cells. (A) Expression of galectin-3. Cells (BT549_{par}) were stably transfected with control vector (BT549_{neo}) or vectors containing galectin-3 (BT549_{gal-3}). Three clones (#11914, #11811, and #11913) were selected. Cell lysates containing equal amount of protein (20 µg) were separated by 12% SDS-PAGE and immunoblotted with rabbit polyclonal antibodies against galectin-3 (Gal-3). Cells were treated with 100 ng/ml TRAIL for 2 hr and survival was analyzed by morphological evaluation (B), crystal violet staining (C), or Trypan blue exclusion assay (D).

Figure 2. TRAIL-induced necrosis and apoptosis. BT549_{neo} cells were treated without (A) or with 100 ng/ml TRAIL (B) for 4 hr. BT549_{gal-3#11914} cells were treated without (C) or with 100ng/ml TRAIL (D) for 4 hr. Cells were stained with annexin V-FITC and propidium iodide (PI), followed by flow cytometric analysis. Apoptotic cells were detected by annexin V-FITC staining (top right quadrant) and necrotic cells by PI staining (top left quadrant).

Figure 3. Effect of galectin-3 on the TRAIL-induced capases activation and PARP cleavage. (A) Parental BT549 (BT549_{par}), control vector transfected (BT549_{neo}), or galectin-3 transfected (BT549_{gal-3#11914}) cells were treated with 100 ng/ml TRAIL for 2 hr. Cell lysates were subjected to immunoblotting for caspase-8, caspase-9, caspase-3, or PARP. Antibody against caspase-8 detects inactive form (54, 55kDa), and cleaved intermediates (41, 43 kDa). Anti-caspase-9 antibody detects both inactive form (46 kDa) and cleaved intermediate (37 kDa). Anti-caspase-3 antibody detects inactive form (32kDa), cleaved intermediate (20 kDa) and active form (17 kDa). Immunoblots of PARP show the 116 kDa PARP and the 85 kDa

apoptosis-related cleavage fragment. Actin was used to confirm the amount of proteins loaded in each lane. (B) BT549_{neo} (○) or BT549_{gal-3#11914} (●) cells were treated with 100 ng/ml TRAIL for various times (0-90 min) and then Caspase-8, -9, and -3-like activity were determined by enzyme kinetics assay as described in Materials and Methods.

Figure 4. UV irradiation-induced proteolytic cleavage of PARP. BT549_{par}, BT549_{neo}, or BT549_{gal-3#11914} cells were exposed to UV-B (3000 joules/m²). Various times (4-12 hr) after UV irradiation, cells were harvested. Lysates containing equal amounts of protein (20 µg) were separated by SDS-PAGE and immunoblotted. C, untreated control cells. Actin was used to confirm the equal amount of proteins loaded in each lane.

Figure 5. Autoradiograph of apoptosis-related gene expression array. Purified mRNA from BT549_{neo} and BT549_{gal-3#11914} cells was reverse transcribed and hybridized on Human Apoptosis Expression Array membranes. The membranes were autoradiographed.

Figure 6. Comparison between intracellular levels of DR4, DR5, DcR2, DAP3, and FADD in BT549_{neo} and that in BT549_{gal-3#11914}. Cell lysates containing equal amount of protein (20 µg) were separated by SDS-PAGE and immunoblotted as described in Materials and Methods.

Figure 7. The intracellular levels of total, reduced (GSH), and oxidized (GSSG) glutathione content in BT549_{par}, BT549_{neo} and BT549_{gal-3#11914}.

Figure 8. Intracellular level of FLIPs in BT549_{neo} and BT549_{gal-3#11914} cells and effect of TRAIL on FLIPs. Cells were treated with various concentrations of TRAIL for 2 hr. Cell lysates

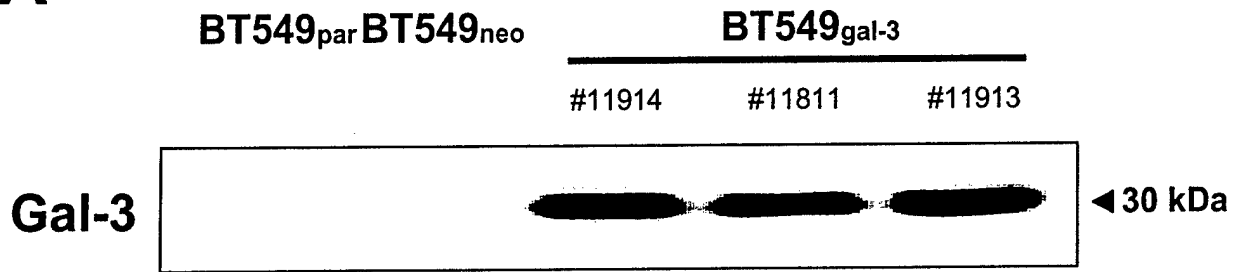
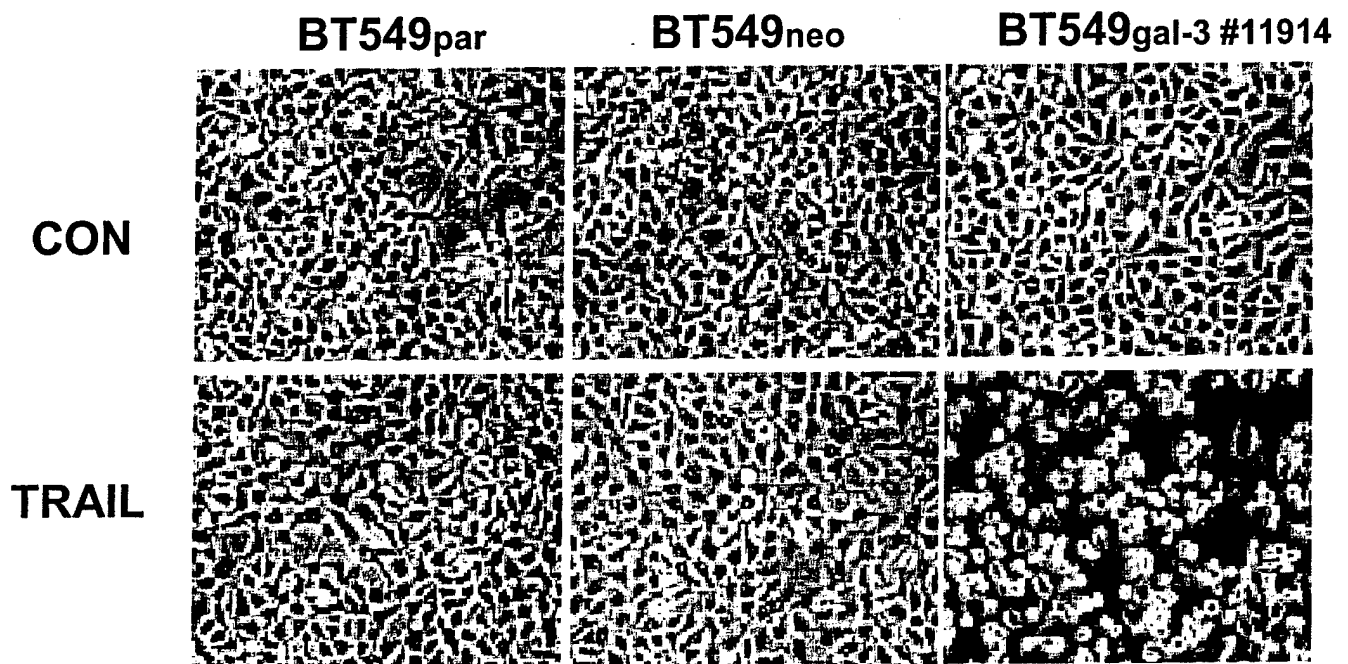
containing equal amount of protein (20 µg) were separated by 12% SDS-PAGE and immunoblotted with anti-FLIPs antibody.

Figure 9. Overexpression of FLIPs in BT549 cells and its effect on TRAIL-induced cytotoxicity and PARP cleavage. (A) Cells were stably transfected with vectors containing Flag-tagged FLIPs cDNA. Lysates containing equal amounts of protein (20 µg) were separated by 12% SDS-PAGE and immunoblotted with anti-FLIPs antibody. Actin is shown as an internal standard. (B) BT549_{par}, BT549_{neo}, BT549_{gal-3#11914}, and three clones of FLIPs overexpressed cells (FLIPs#7, FLIPs#11, FLIPs#12) were treated with 100 ng/ml TRAIL for 2 hr and then their survival percentages were assessed by Trypan Blue dye exclusion method. Survival fractions are shown as the mean ± standard error from triplicate experiments. (C) Cells were treated with TRAIL as described in (B) and TRAIL-induced proteolytic cleavage of PARP was analyzed. Immunoblots of PARP show the 116 kDa PARP and the 85 kDa apoptosis-related cleavage fragment. Actin was used to confirm the amount of proteins loaded in each lane.

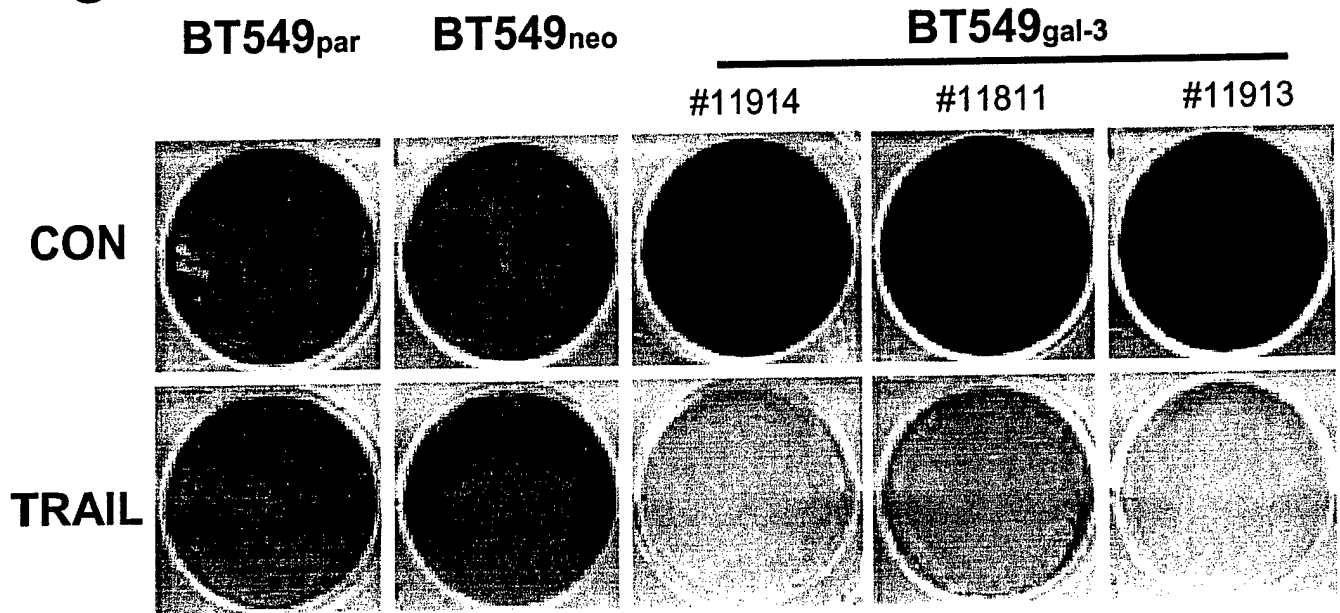
Figure 10. Effect of reconstitution of galectin-3 on Akt S473 phosphorylation. Cell lysates from BT549_{par}, BT549_{neo}, BT549_{gal-3#11914}, BT549_{gal-3#11811}, or BT549_{gal-3#11913} were subjected to Immunoblotting with anti-Akt antibody or anti-phospho-S473 Akt antibody.

Figure 11. Effect of overexpression of myr-Akt or wt-Akt. BT549_{gal#11914} cells were infected with adenoviral vectors containing a constitutively activated Akt (Ad.myr-Akt) or wild-type Akt (Ad.wt-Akt) at various MOIs (1, 10, 100). Cells were treated with 100 ng/ml TRAIL for 2 hr. (A) Cell lysates containing equal amounts of protein were immunoblotted with anti-phospho-Ser-473 Akt antibody. Total Akt was detected with anti-Akt antibody. Actin was immunoblotted to

confirm the equal amount of protein loaded in each lane. **C**, untreated control cells. **T**, TRAIL treated cells. **(B)** Cell survival was analyzed by Trypan blue exclusion assay.

A**B****Fig. 1A & B**

C



D

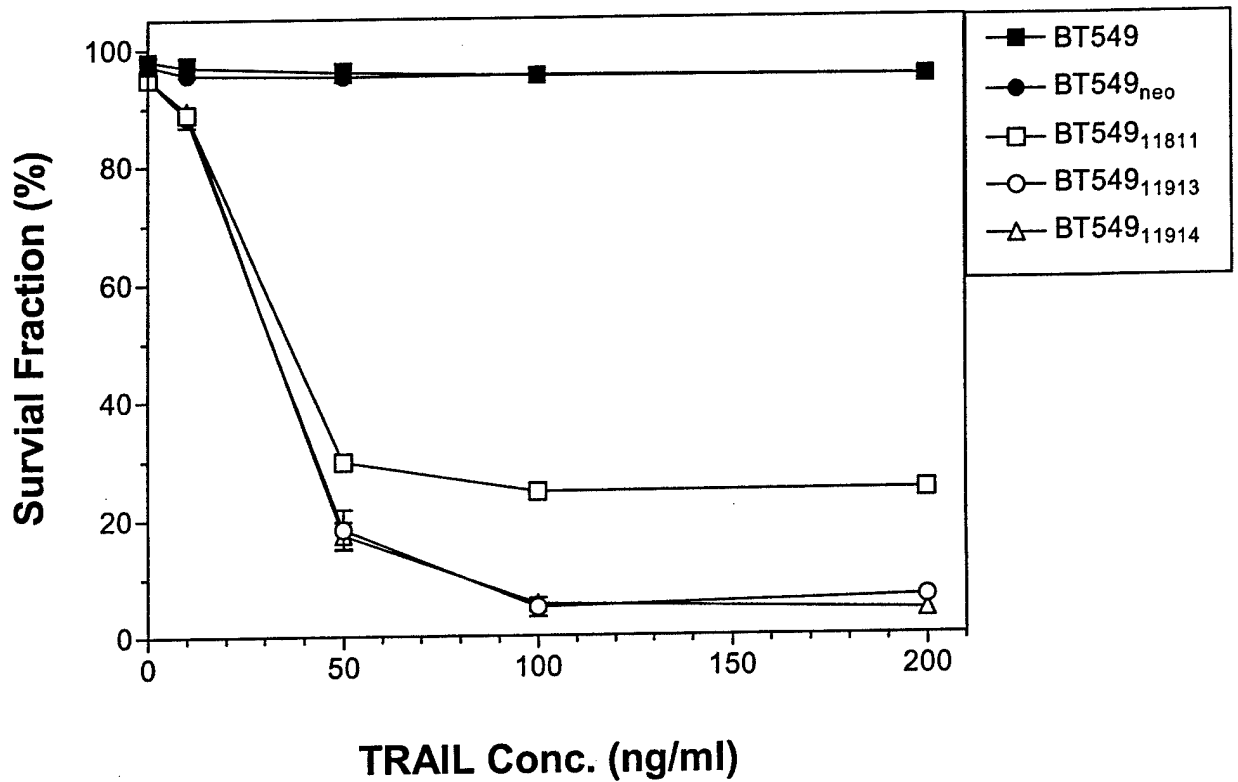


Fig. 1C & D

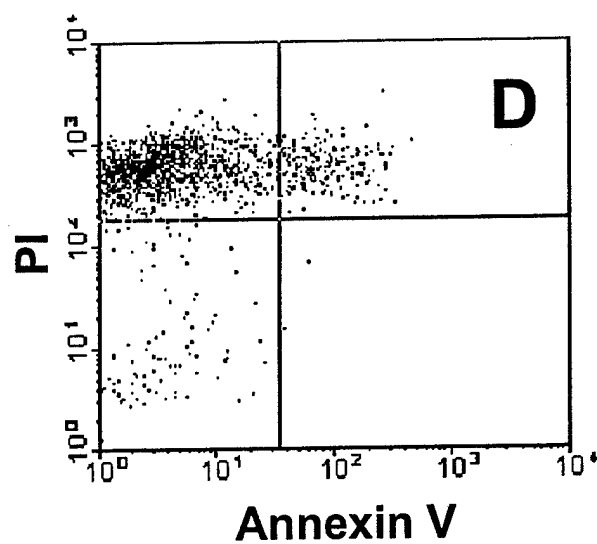
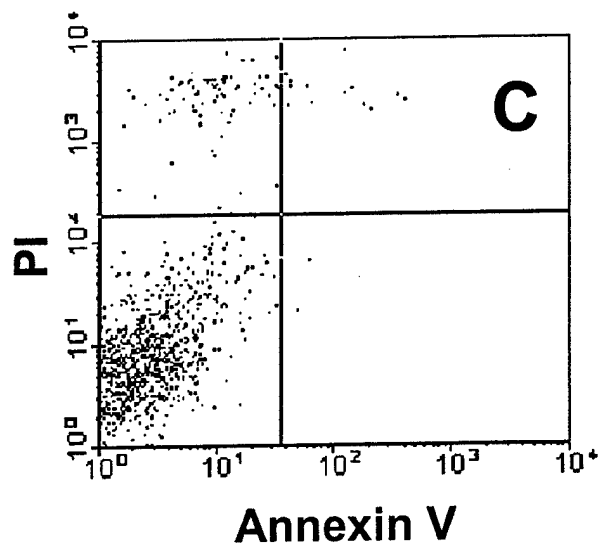
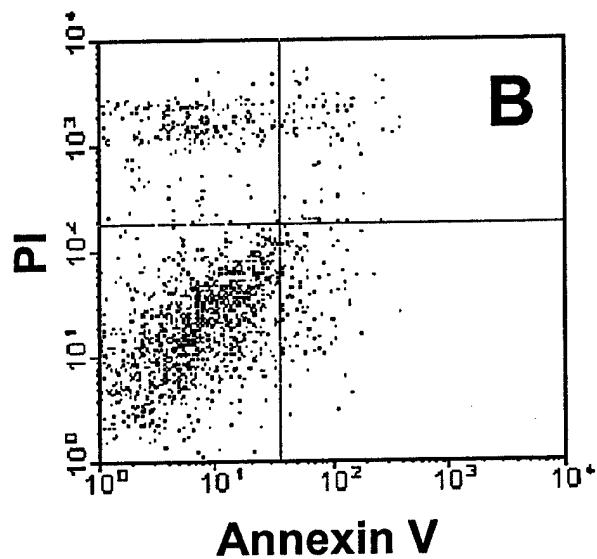
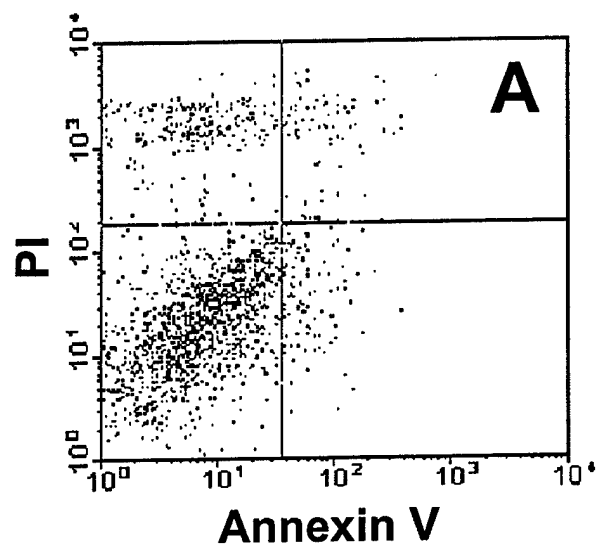


Fig. 2

A

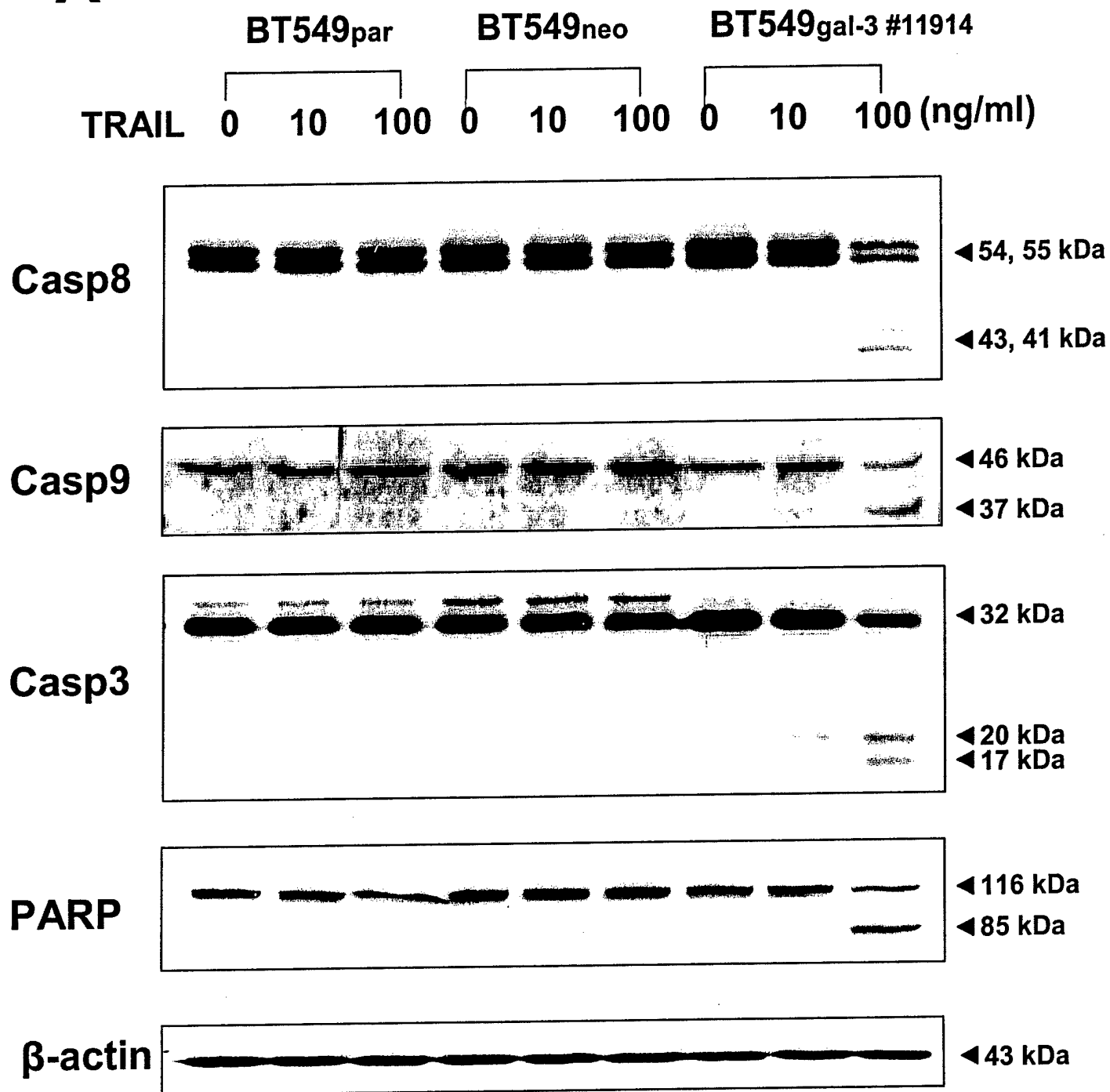
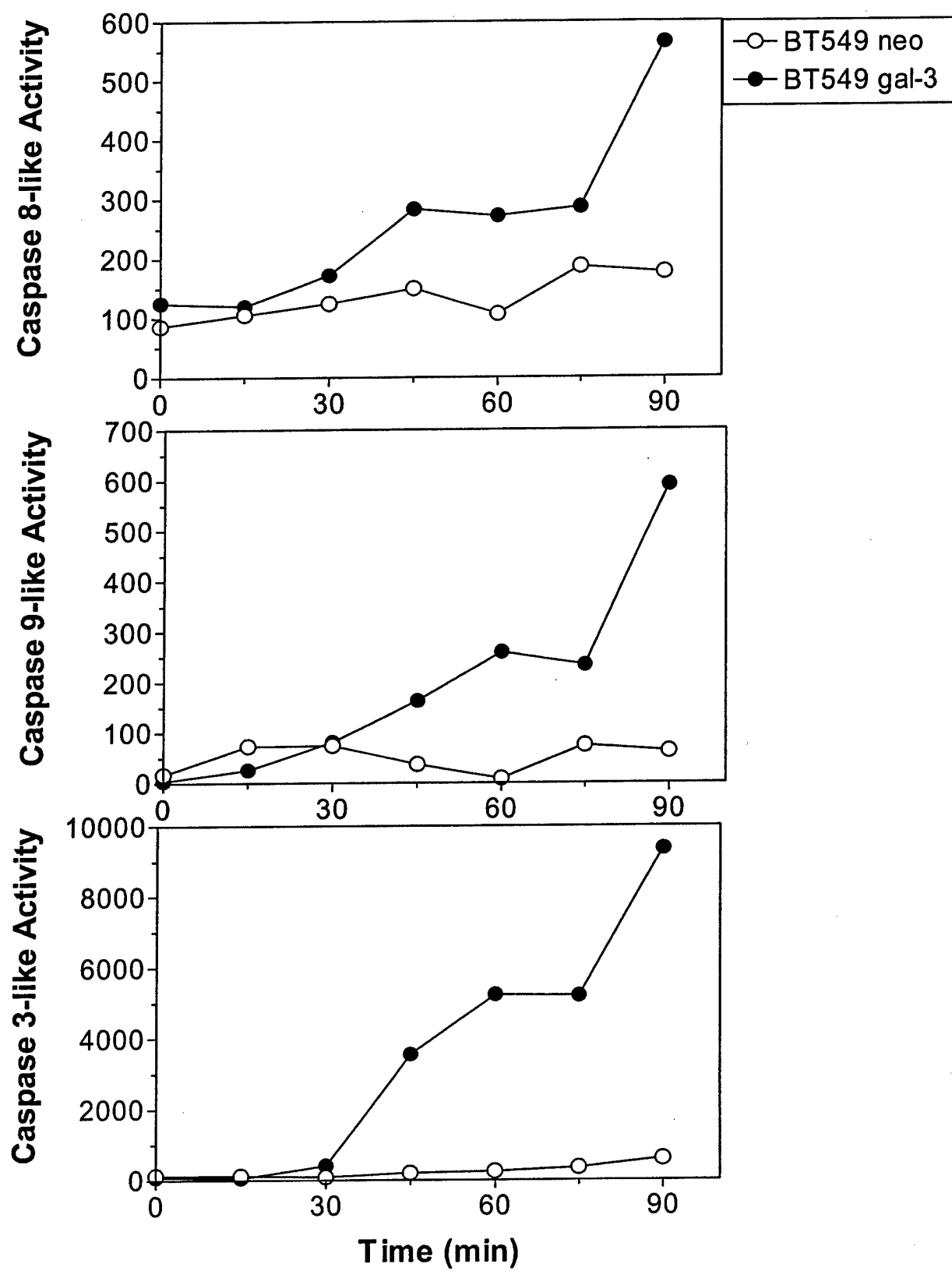


Fig. 3A

B**Fig. 3B**

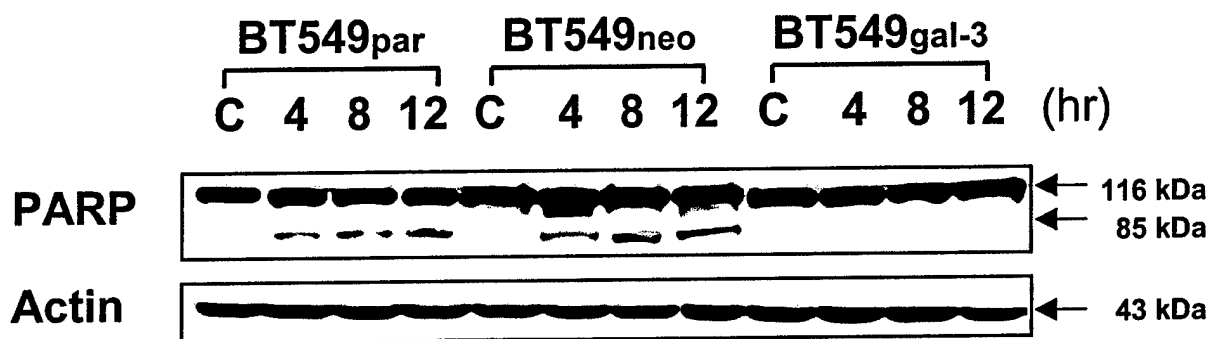


Fig. 4

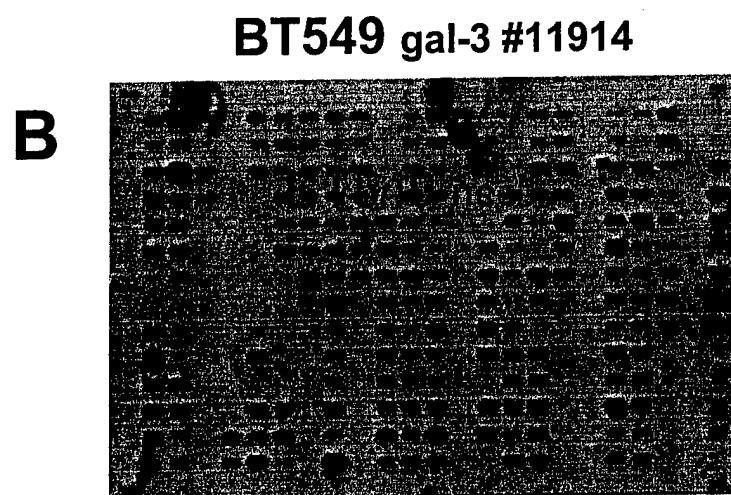
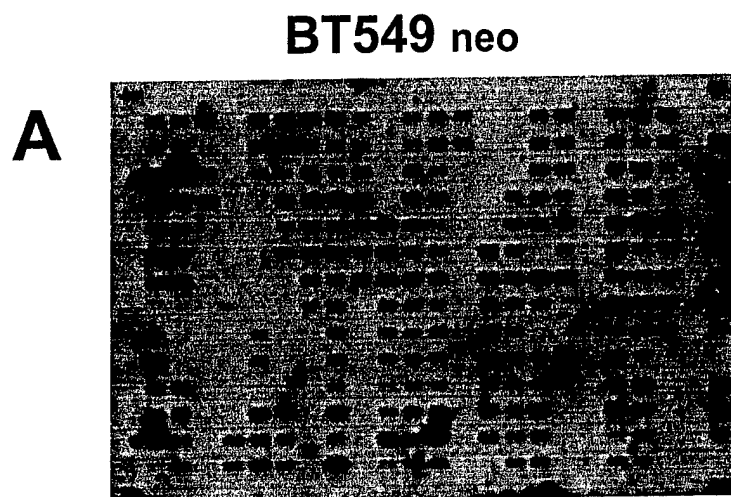


Fig. 5

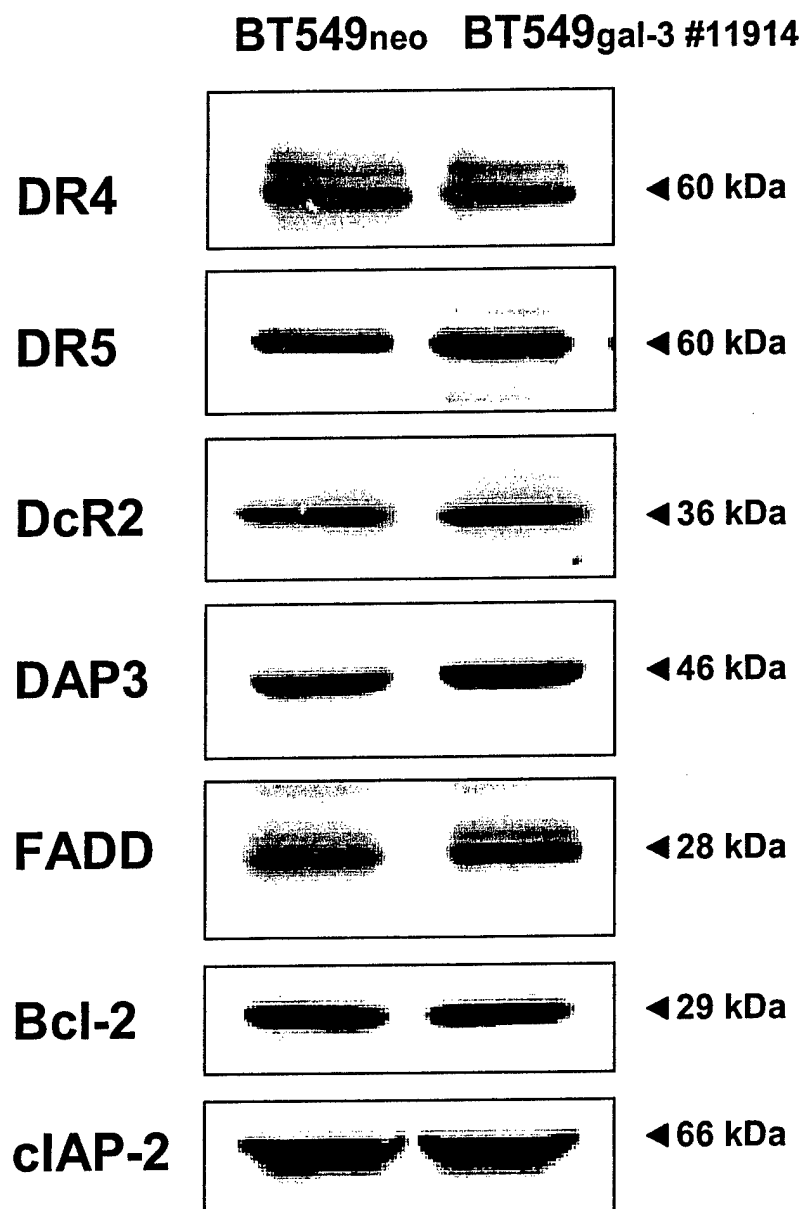


Fig. 6

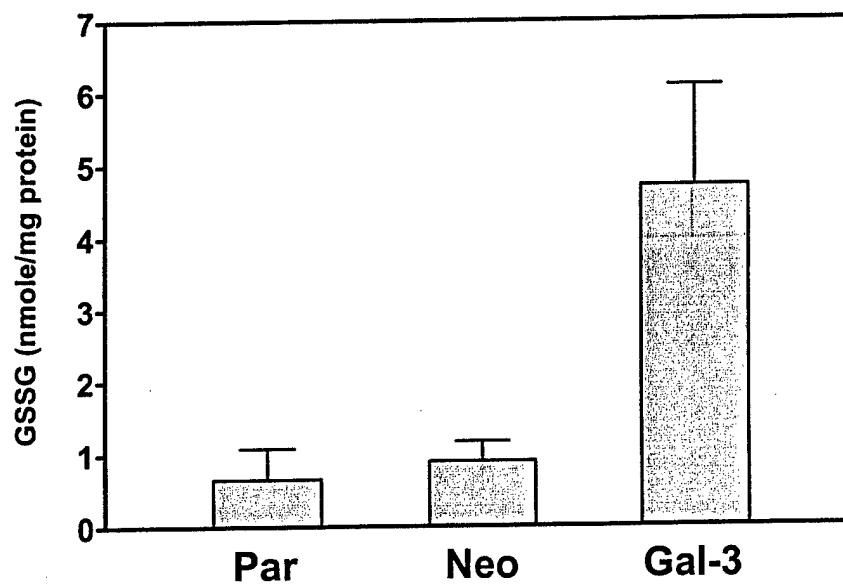
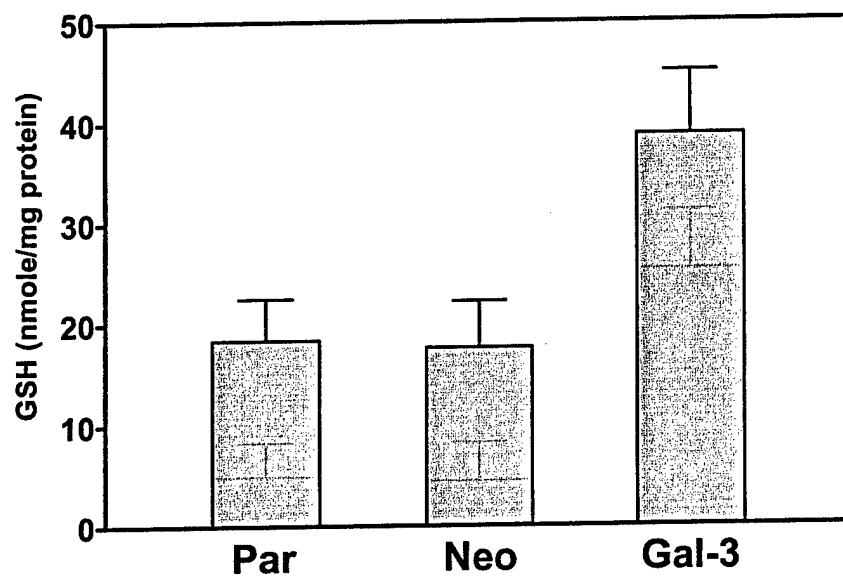
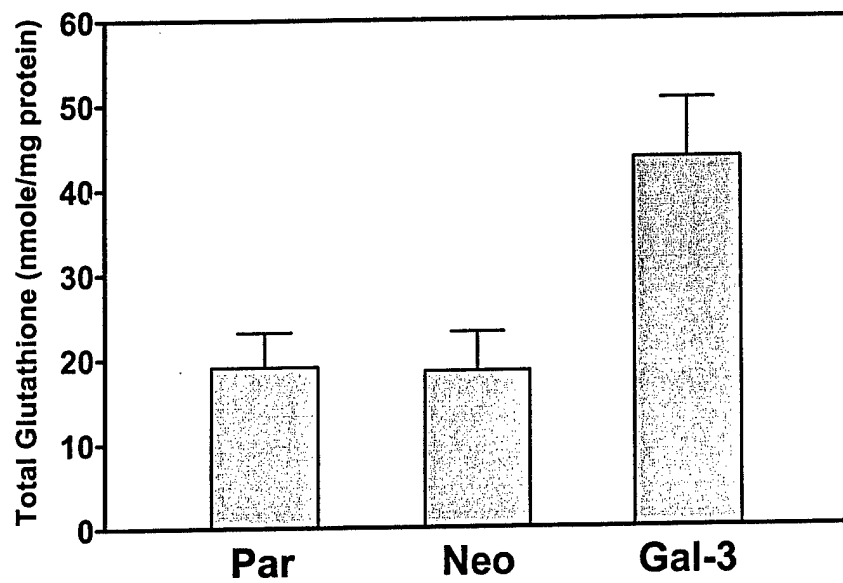


Fig. 7

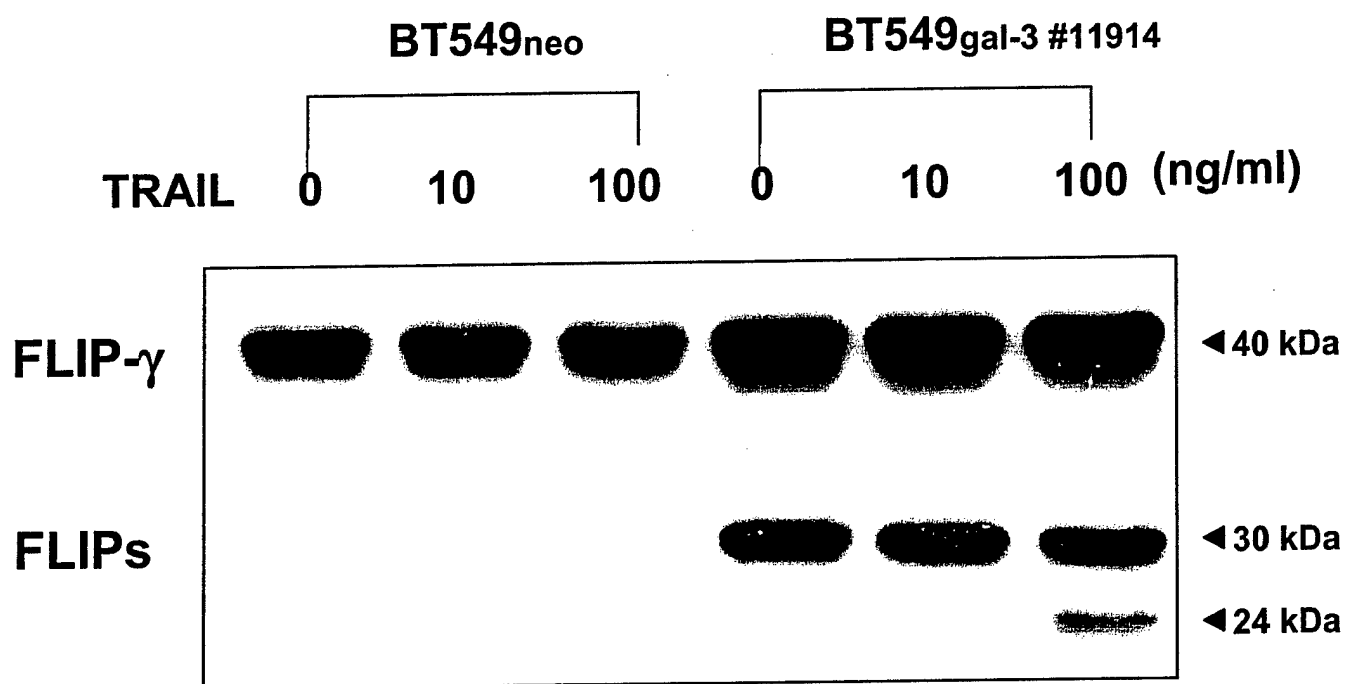


Fig. 8

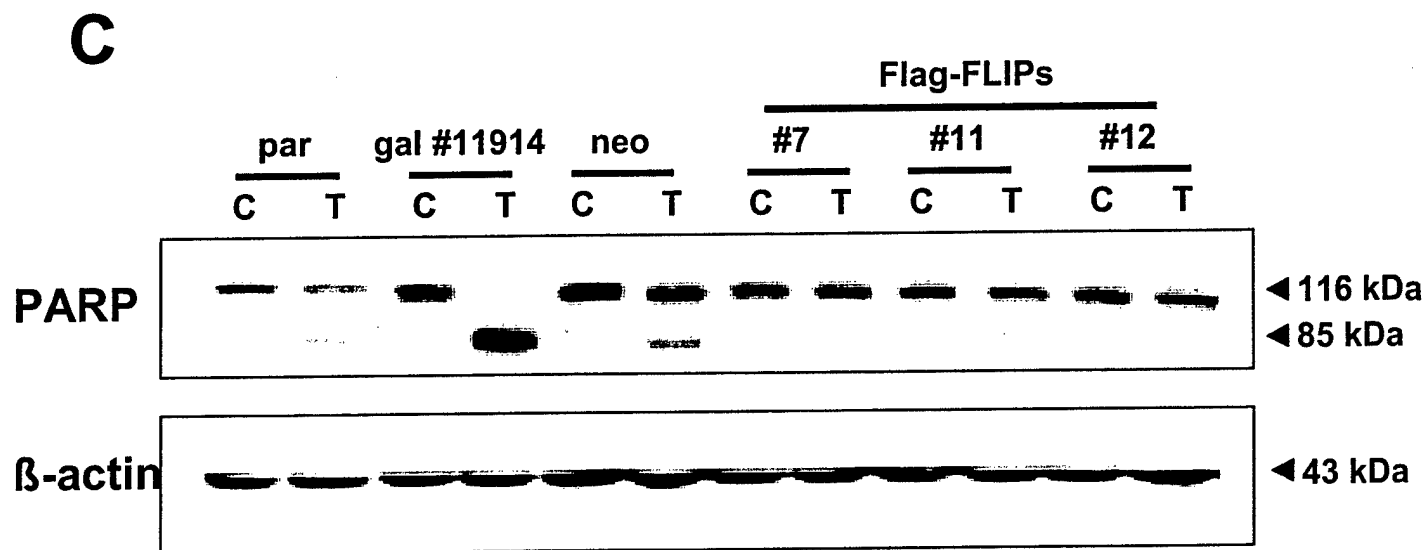
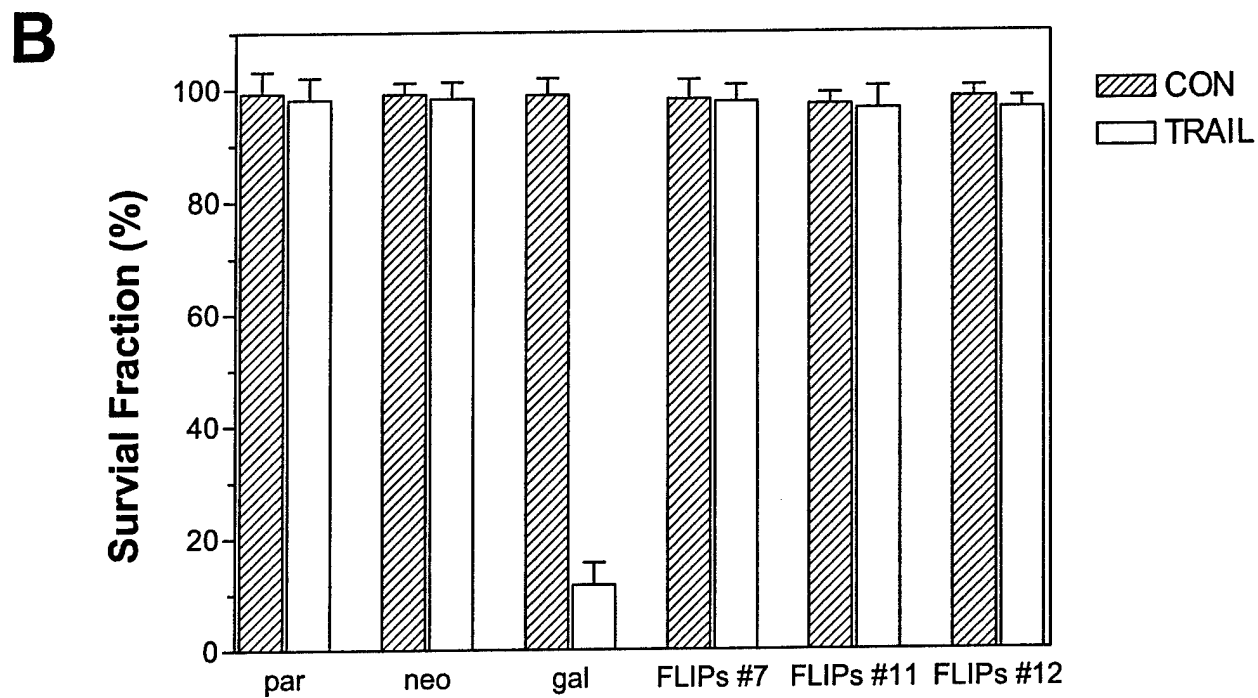
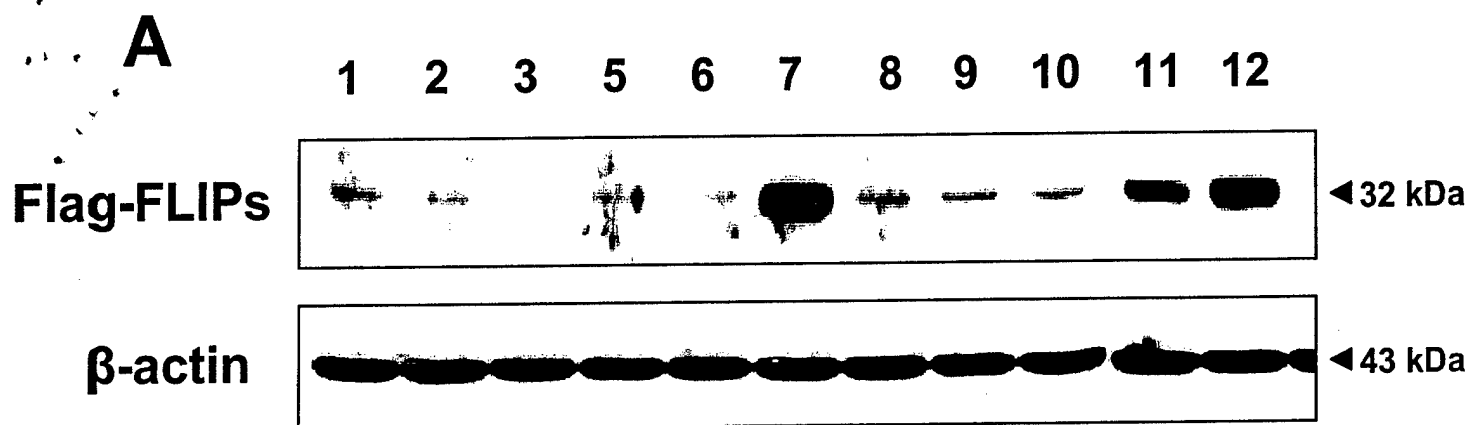


Fig. 9

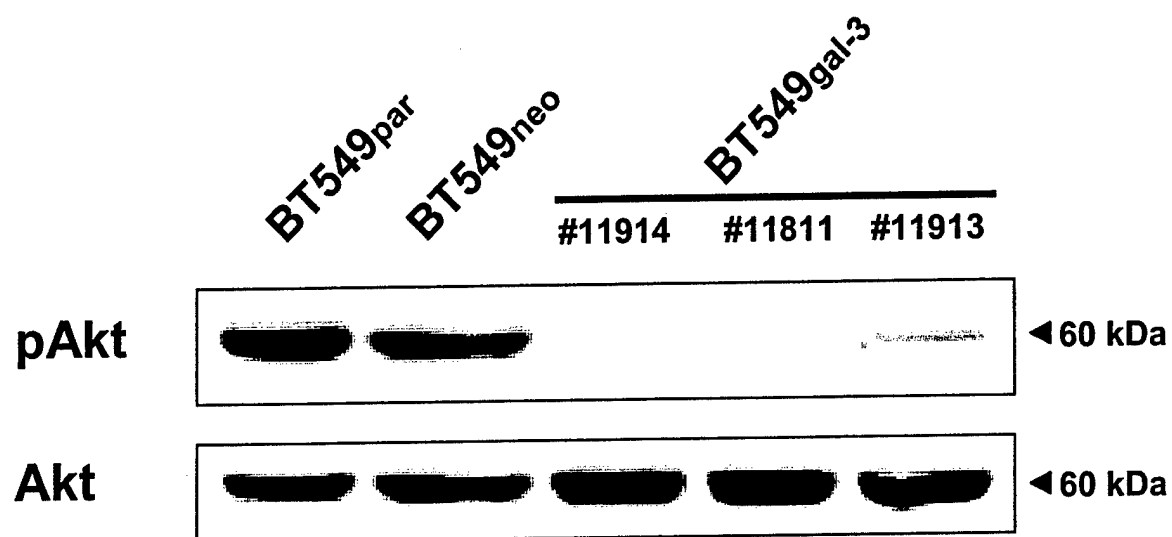


Fig. 10

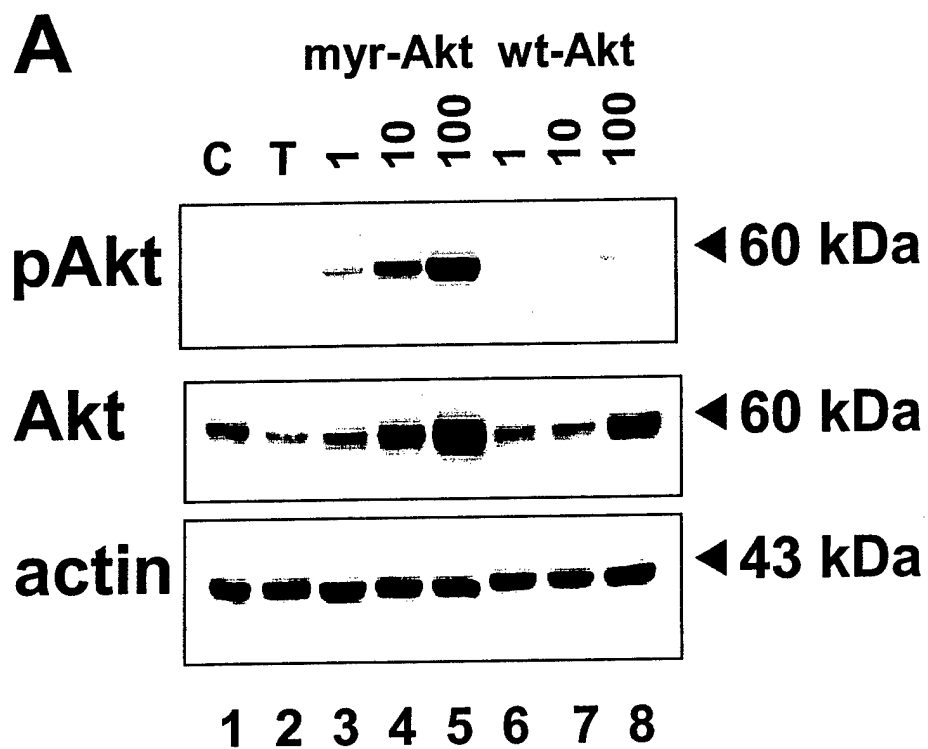


Fig. 11A

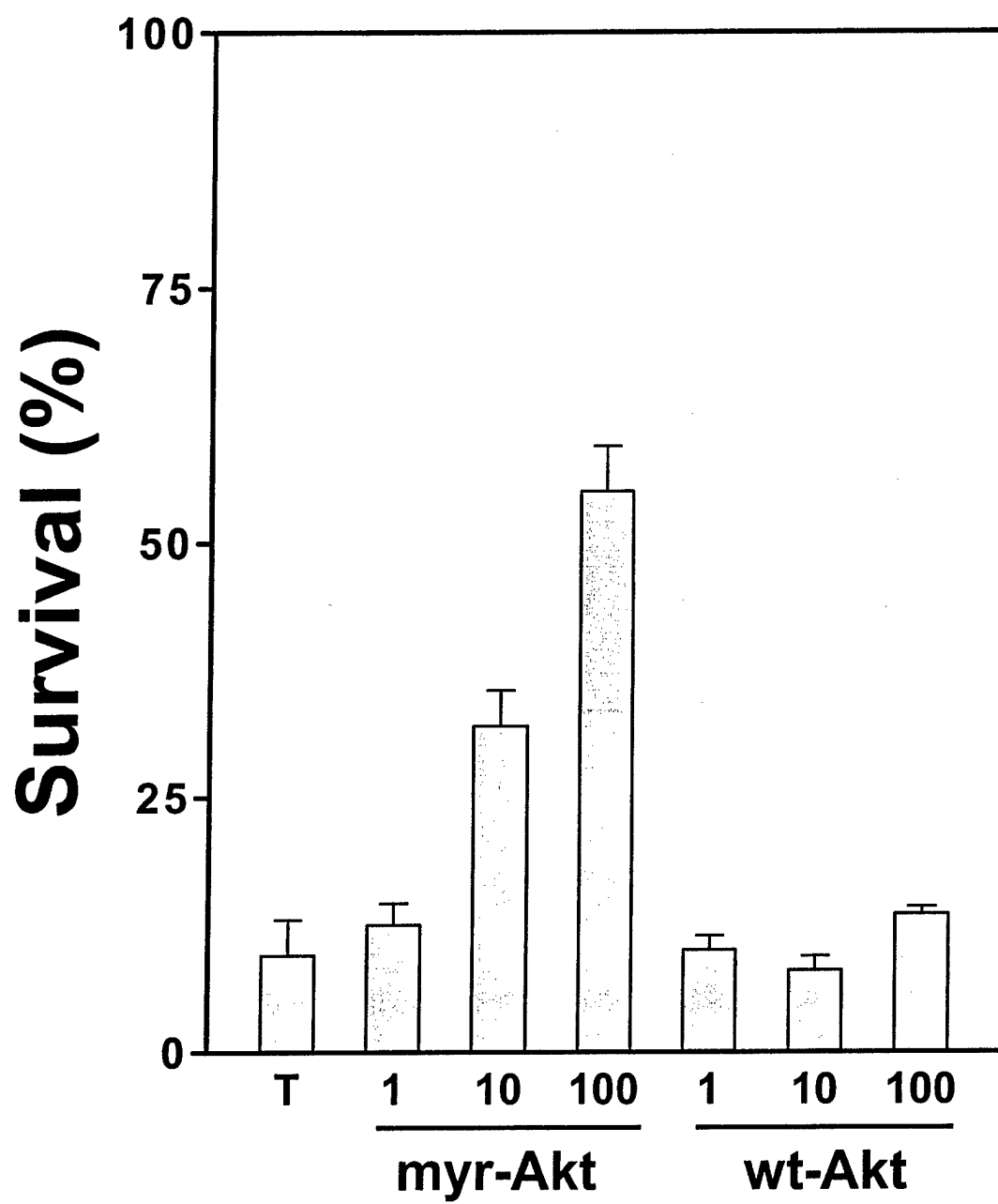


Fig. 11B